Antioxidant properties of 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid (8-8-diferulic acid, non-cyclic form)†

MT Garcia-Conesa,*[1] PD Wilson,[1] GW Plumb,[1] J Ralph and G Williamson[1]

[1] Biochemistry Department, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK
[2] US Dairy Forage Research Centre, Agricultural Research Service, US Department of Agriculture, 1925 Linden West Drive, Madison, Wisconsin, 53706, USA

Abstract: 8-8-Diferulic acid (non-cyclic form) (4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid) was chemically synthesised and the antioxidant properties were assessed using (a) inhibition of ascorbate/iron-induced peroxidation of phosphatidylcholine liposomes, and (b) scavenging of the radical cation of ABTS (2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate)) relative to the water-soluble vitamin E analogue, Trolox C. The structure of the chemically synthesised 8-8 dimer was confirmed by ^{1}H-NMR and mass spectrometry. Its absorption properties in ethanol were: λ_{max1}: 320 nm; λ_{max2}: 287 nm; λ_{min}: 256 nm; $\varepsilon_{\lambda max1}$ ($M^{-1}cm^{-1}$): 14 200 ± 1700 and $\varepsilon_{\lambda max2}$ ($M^{-1}cm^{-1}$): 14 300 ± 1300. The 8-8 dimer showed the best antioxidant properties in the aqueous phase assay among the esterified dimers present in plant cell walls. Like the other ferulic dimers, 8-8-diferulic acid is a better inhibitor of lipid peroxidation than ferulic acid on a molar basis. Some of the factors possibly involved in the antioxidant effect of these compounds are: number of free hydroxyl groups in the molecule, stability of transient radical and partition coefficient.

Keywords: ferulic acid; diferulic acids; 8-8-diferulic acid; antioxidant capacity

INTRODUCTION

trans-Ferulic acid is found in significant quantities and mainly ester-linked to polysaccharides in plant cell walls of the family Graminaceae and Chenopodiaceae.[1,2] Oxidative coupling of plant cell wall ferulate esters via the action of peroxidases produces several regio-isomeric diferulates that can be chemically synthesised.[3] The radical-coupling products are predominantly 8-5-BendiFA (*trans*-5-[(*E*)-2-carboxyvinyl]-2- (4-hydroxy-3-methoxy- phenyl)-7-methoxy- 2,3-dihydrobenzofuran- 3-carboxylic acid) and 8-*O*-4-diFA((*Z*)-*β*- {4-[(*E*)-2-carboxyvinyl]- 2-methoxy-phenoxy}-4- hydroxy-3-methoxycinnamic acid), whereas 5-5-diFA ((*E*,*E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid) and 8-8-diFA (4,4′-dihydroxy-3,3′-dimethoxy-*β*,*β*′-bicinnamic acid) are present in lower concentrations. A significant proportion of these esterified dimers have now been quantified in cell walls of several plant materials.[3–6]

Ferulic acid has effective antioxidant properties with potential applications in the pharmaceutical and food industries.[7] The antioxidant capacity of two chemically-synthesised ferulic dimers, 5-5 and 8-5-diFA, has been reported.[8] One of the main ferulic dimers present in wheat bran, 8-*O*-4-diFA, was purified by preparative chromatography and its antioxidant capacity examined.[9] The ferulic acid dimers are more effective inhibitors of lipid peroxidation than ferulic acid on a molar basis. The ability of these phenolic compounds to scavenge radicals such as ABTS$^{\cdot}$ in the aqueous phase seems to be related to the existence of a full conjugation system in the molecule.[8,9]

In order to further understand the ability of ferulic acid and its dimers to act as antioxidants, we have synthesised 8-8-diferulic acid (non-cyclic form) following the method of Ralph *et al*[3] and assayed its antioxidant properties in aqueous and lipid phases. The results are compared to those obtained previously for other ferulic acid dimers and for ferulic acid itself.[8,9] The possible influence of lipophilicity (expressed as partition coefficient), number of hydroxyls and transient radical stability on antioxidant capacity is also discussed.

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* Correspondence to: Maria Teresa Garcia-Conesa, Biochemistry Dept, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK.

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EXPERIMENTAL

Materials

8-8-Diferulic acid (non-cyclic form) (4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid) was synthesised by the method of Ralph *et al*.[3] The final product was confirmed by ^{1}H NMR and mass spectrometry (EIMS (probe) 70 eV *m/z* (rel int): 386 (0.2), 368 (17.9), 342 (19.4), 324 (1.5), 298 (100), 281 (19.9), 237 (20.8), 162 (25.7), 137 (55.6)). On HPLC the purity of the final product was >90% (absorbance at 280 nm). Trolox C and ABTS were supplied by Aldrich Chemical Company (Dorset, UK). Phosphatidyl choline from frozen egg yolk in ethanol (100 mg ml^{-1}) was purchased from Sigma (type XII-E) and was 99% pure. All other chemicals were of AnalaR or HPLC grade purity.

Determination of the absorption coefficients

The absorption coefficients for the 8-8 dimer were obtained from the corresponding solution of the compound in ethanol (AnalaR, 99.7–100%, BDH).[8]

Lipid and aqueous phases antioxidant activity

Scavenging of ABTS radicals

The TEAC (Trolox C Equivalent Antioxidant Capacity) value was measured by the method of Salah *et al*.[10] Values are expressed relative to a standard of Trolox C, the water soluble analogue of vitamin E (the concentration of the compounds tested was 0.5 mM). The extent of quenching of the ABTS radical was measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Positive controls were performed as described previously.[8]

Inhibition of lipid peroxidation

Peroxidation of phospholipid liposomes was performed as described previously with a range of 8-8-diferulic acid concentrations.[11] Results are expressed as IC_{50} (μM concentration at which 50% of lipid peroxidation inhibition is reached) where 100% inhibition is baseline peroxidation of liposomes without added iron/ascorbate, and 0% inhibition is peroxidation of liposomes with added iron/ascorbate. Calculation of IC_{50} value was performed by fitting a third order polynomial curve to the data.

Measurement of the partition coefficient (log P)

The lipophilicity of a compound is usually expressed as a partition coefficient between 1-octanol and water. For ionisable compounds, pH affects lipophilicity depending on the pK_a value(s). For the measurement of log *P* for ferulic acid and ferulic dimers we have used the conventional method of shake-flasks with octanol in buffer.[12] Three aqueous buffer solutions were used: 20 mM potassium chloride + HCl (pH 2.0), 20 mM acetic acid + NaOH (pH 5.0) and 5 mM phosphate buffer (pH 7.0). Each buffer solution was saturated with octanol.

Ferulic acid and its dimers were prepared in water-saturated octanol (10^{-3}–10^{-5} M), mixed with the buffer solutions and kept at 37°C for 16 h. The final concentration of the compounds in the water phase was determined by HPLC/Diode Array detection.[13] The partition coefficient (log *P*) was calculated according to the equation:

$$P = (C_{\text{octanol}} - C_{\text{water}})/C_{\text{water}}$$

where C_{octanol} is the initial concentration of the compound in the octanol and C_{water} is the final concentration of the compound in the water.

RESULTS AND DISCUSSION

Absorption coefficients

The UV spectrum and structure of 8-8-diferulic acid (non-cyclic) in ethanol is presented in Fig 1. This ferulic dimer exhibits two absorption maxima (λ_{max}) at 320 nm and 287 nm (Table 1). The addition of water to the ethanol results in a red shift of the spectrum (λ_{max} = 323–325 nm) and an increase of the absorption coefficient at the maximum wavelength ($\varepsilon_{\lambda max}$ = up to 20 000 M^{-1} cm^{-1}).

Aqueous and lipid phase antioxidant activity of 8-8-diferulic acid

The results of the antioxidant tests for the 8-8 coupled dimer are shown in Table 2. 8-8-Diferulic acid (non-cyclic form) is the best antioxidant in the aqueous phase. It exhibited a higher TEAC value than the other ferulic dimers tested (between 1.49 and 2.60) and twice the value of ferulic acid (1.96).[8,9] The activity in this assay depends partially on the number of free hydroxyl groups of a molecule and on its ability to transfer a hydrogen atom to a reactive radical R$^{\cdot}$.[14] The dissociation energy of the O–H bond depends on the stabilisation of the transient radical formed. The resonance stabilisation of the ferulic acid radical has been described elsewhere.[7]

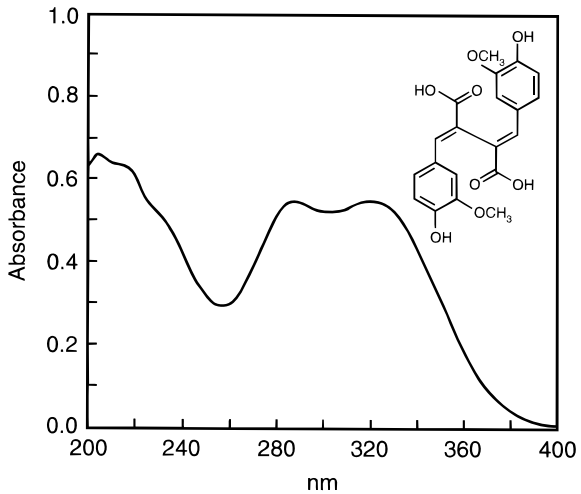

Figure 1. Absorption spectrum and structure of 8-8-diferulic acid (non-cyclic form).

Table 1. Absorption maxima (λ_{max}) and absorption coefficients for ferulic acid dimers in ethanol (mean ± SD, $n = 3$)

	λ_{max1} (nm)	λ_{max2}(nm)	λ_{min} (nm)	$\varepsilon_{\lambda max1}$ ($M^{-1}cm^{-1}$)	ε_{280} ($M^{-1}cm^{-1}$)
8-8-diFA (non-cyclic)	320.0	287.0	256.0	14 200 ± 1700	14 300 ± 1300
5-5-di-FA[a]	324.0	—	273.5	29 800 ± 850	19 100 ± 2000
8-5-bendiFA[a]	323.5	—	265.0	21 000 ± 800	10 000 ± 100
8-*O*-4-diFA[b]	323.0	—	258.0	24 800 ± 2100	19 700 ± 1100

[a] Ref 8.
[b] Ref 9.

Some of the possible resonance forms of the 8-8-diferulic radical are presented in Fig 2. The 8-8 dimer has two free phenolic hydroxyl groups and two complete ferulic moieties which could explain the superior antioxidant capacity of the molecule due to a higher degree of conjugation and larger delocalisation of the unpaired electron.

The 8-5 dimer, which has one phenolic hydroxyl and one conjugation system interrupted by the β-*O*-4 ring, also has the lowest antioxidant capacity in the aqueous phase among the ferulic acid dimers.[8] The delocalisation of the unpaired electron in the radical is shown in Fig 3. The results obtained with 8-8 and 8-5 dimers suggest that antioxidant capacity is increased with more hydroxyl groups and longer uninterrupted conjugation in the molecule. However, this does not hold true for the 8-*O*-4 and the 5-5 dimer. The former has one hydroxyl and one complete conjugation system (Fig 4) and the latter has two phenolic hydroxyls and two complete conjugation systems (Fig 5) but their TEAC values (2.6 and 2.2 respectively) are slightly higher than the value for ferulic acid and lower than the 8-8 dimer value, suggesting that other factors influence the antioxidant capacity of these compounds. The different types of linkages between the two moieties may affect the stability of the transient radical and therefore the antioxidant capacity of the molecules.

Table 2. Antioxidant properties of ferulic acid and ferulic acid dimers (mean ± SD, $n = 3$)

Compound	*TEAC*[a]	IC_{50}(μM)
8-8-diFA	4.00 ± 0.20	14.0 ± 0.7
Ferulic acid[b]	1.96 ± 0.01	26.6 ± 0.5
5-5-diFA[b]	2.19 ± 0.01	6.9 ± 0.7
8-5-bendiFA[b]	1.49 ± 0.02	9.1 ± 0.5
8-*O*-4-diFA[c]	2.60 ± 0.10	14.0 ± 0.6

[a] Relative to the antioxidant activity of Trolox C (mM).
[b] Ref 8.
[c] Ref 9.

In the lipid phase, the antioxidant capacity of 8-8-diferulic acid was similar to the activity of the 8-*O*-4 dimer although it was not as effective as the 5-5 and the 8-5 dimers. All the ferulic acid dimers tested are more effective in the lipid phase than the monomeric acid.[8,9] In the lipid peroxidation test, phosphatidyl choline microsomes are oxidised in the presence of iron/ascorbate to form lipid peroxides and malondialdehyde (MDA). The assay measures the ability of a compound to reduce the formation of MDA. Therefore, in addition to the structural properties of the dimers, their partitioning between the two phases (water/phospholipid liposomes) may play a role in their antioxidant effect.

Partition coefficient (log *P*)

The log *P* of ionisable compounds changes with pH. For measuring true log *P* values it is important to

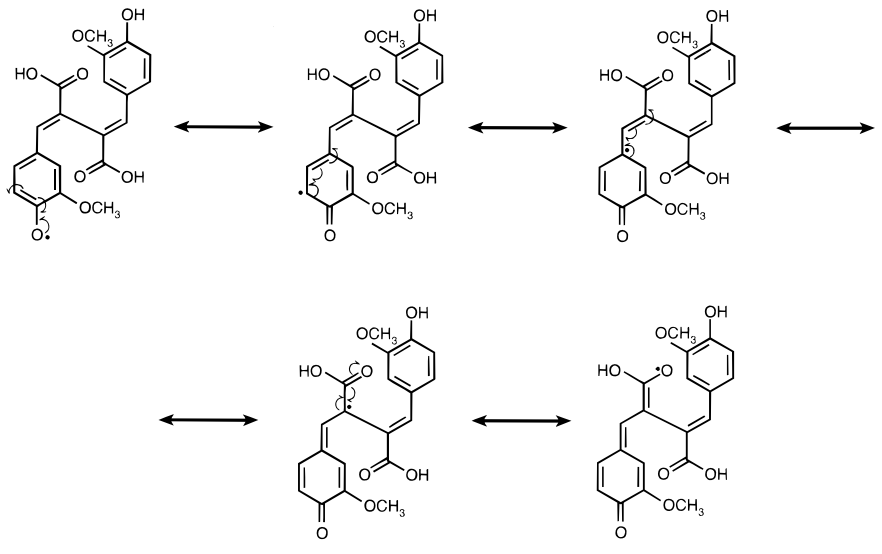

Figure 2. Resonance forms of 8-8-diferulic acid.

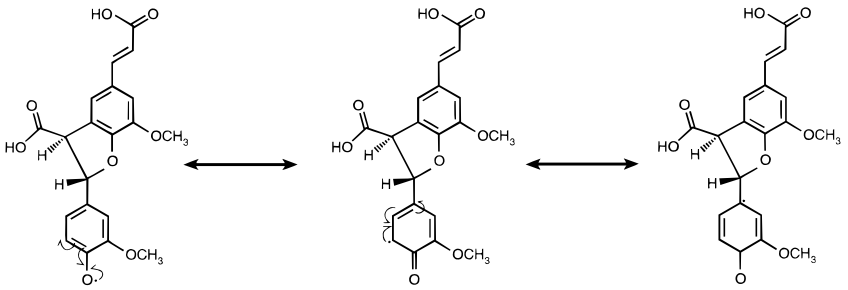

Figure 3. Resonance forms of 8-5-benzofuran diferulic acid.

ensure that the water phase is buffered to a pH lower than the pK_a of the compound so that the compound is present mainly in the non-dissociated form. The pK_a of the carboxylic acid group of hydroxycinnamic acids is in the range 4–5.[15] The pK_a values for the ferulic dimers have not yet been determined.

In order to evaluate the role of lipophilicity on the antioxidant effectiveness of ferulic dimers, the partition coefficient of ferulic acid and its dehydrodimers in an octanol/water system at three pH values were measured. The results are presented in Table 3. At the lowest pH (2.0), the compounds should be essen-

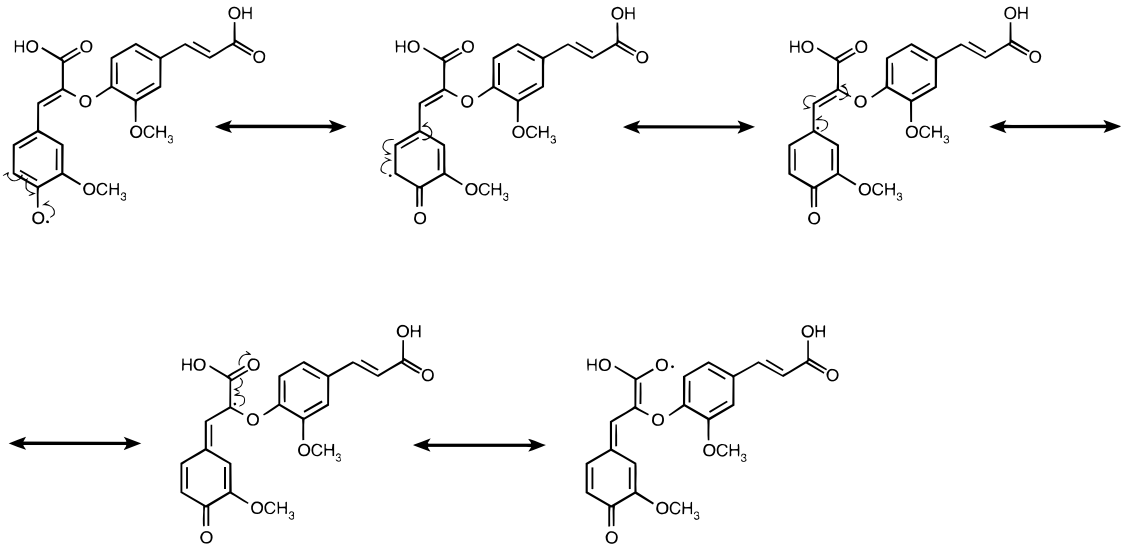

Figure 4. Resonance forms of 8-O-4-diferulic acid.

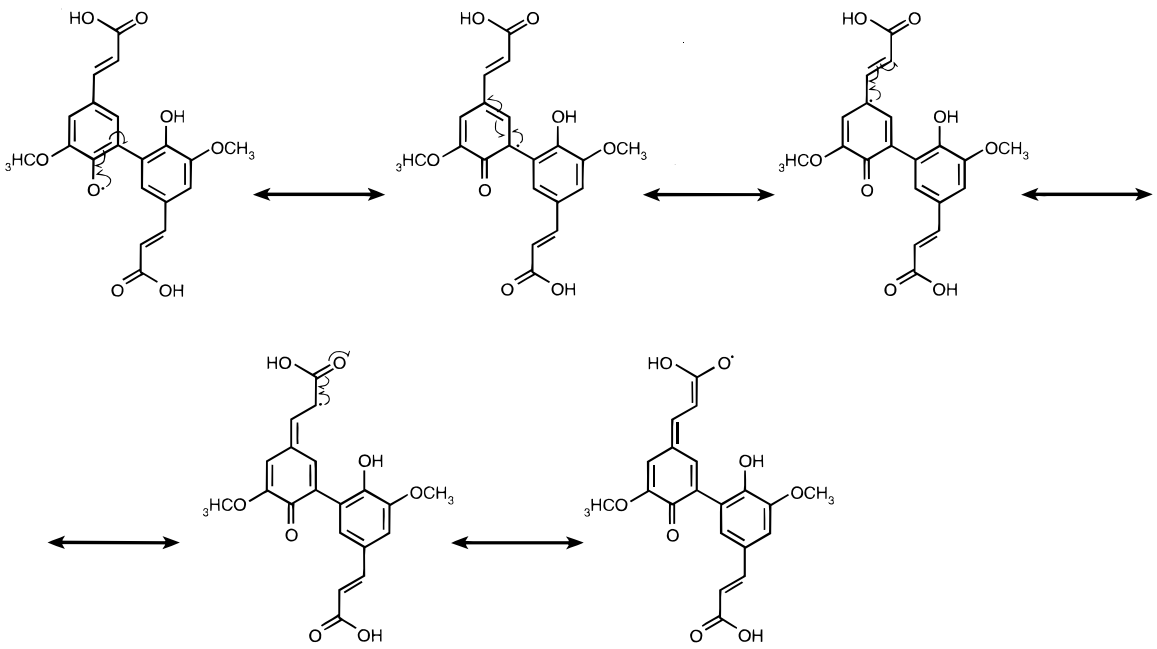

Figure 5. Resonance forms of 5-5-diferulic acid.

Compound	*log P (pH: 2.14)*	*log P (pH: 5.0)*	*log P (pH: 7.0)*
Ferulic acid	1.71 ± 0.14	1.17 ± 0.02	−0.25 ± 0.10
5-5-diFA	2.73 ± 0.09	1.18 ± 0.03	−0.73 ± 0.37
8-5-bendiFA	2.72 ± 0.04	0.57 ± 0.01	−0.11 ± 0.04
8-*O*-4-diFA	2.73 ± 0.22	0.45 ± 0.02	−1.04 ± 0.29
8-8-diFA	1.74 ± 0.06	0.40 ± 0.02	−0.50 ± 0.04

Table 3. Partition coefficient of ferulic acid and its dehydrodimers in octanol/water at three different pH values (mean ± SD, $n = 3$)

tially in the non ionised form. 8-8-Diferulic acid exhibited the same log P value as ferulic acid, whereas the other three dimers tested were more lipophilic than ferulic acid.

The lipid peroxidation test is performed at pH 5.7. Under these conditions all the compounds assayed are partially dissociated and consequently partitioning into the lipid phase is diminished. The log P values measured at pH 5.0 and 7.0 indicate a marked and different increase in the dissociation of each of the compounds probably due to differences in the pK_a of the carboxylic groups. Therefore each of the dimers will partition to a different degree into the organic phase. In the conditions of our assay, the proportion of compound interacting with the lipid phase may be very different for each of the dimers tested. The results obtained on the partition coefficient can not be correlated to the lipid peroxidation inhibition capacity of the dimers. As would be expected, partitioning into the organic phase alone does not explain the differences in the antioxidant capacity of ferulic acid and ferulic dimers in the lipid phase. The ability of these compounds to bind iron ions (present in the system) or to scavenge reactive oxygen species in the water phase (produced by the system iron/ascorbate) may also play a role in the final effect.

CONCLUSIONS

(i) 8-8-Diferulic acid (non-cyclic form) is the most effective antioxidant in the aqueous phase among the dimers of ferulic acid. This can be partially due to a better resonance stabilisation of the molecule and the presence of two phenolic hydroxyl groups.

(ii) In the lipid phase the antioxidant capacity of the 8-8 dimer was similar to the activity of the 8-*O*-4 dimer but the compound was not as effective as the 5-5 and the 8-5 dimers. Ferulic dimers are better inhibitors of lipid peroxidation than ferulic acid.

(iii) Non-dissociated 8-8-diferulic acid has the same partition coefficient as ferulic acid. 5-5, 8-*O*-4 and 8-5 dimers are more lipophilic than ferulic acid.

(iv) In the conditions used for lipid peroxidation, ferulic acid and ferulic dimers are partially dissociated. The proportion of non-dissociated form is different for each of the compounds and therefore the amount of compound partitioning into the lipid phase would also be different. This affects the final antioxidant effect in the lipid phase but alone does not explain the differences observed between ferulic acid and ferulic dimers.

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